An Enzyme-mediated Target-specific Signal Amplifier: Engineered Ascorbate Peroxidase 2/ Antibody-binding Domain Fusion Protein

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01. 16. 2015

Approved by

Advisor

Sebyung Kang

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01. 16. 2015

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Abstract

There are various analytical procedures based on the antigen-antibody interactions and signal amplifications, because antibodies generally give high binding specificity and affinity toward any given target molecules and signal amplification assures the detection of low abundant molecules. We report here the novel approach to substitute conventional horseradish peroxidase (HRP) conjugated secondary antibodies with a recombinant antibody-binding domain (ABD) fused peroxidase. The Z domain, antibody-binding domain of protein A, has high binding affinity and specificity to the Fc region of immunoglobulin G (IgG) and the Engineered ascorbate peroxidase 2 (APEX2) has a capability to catalyze hydrogen peroxides (H2O2) to generate reactive oxygen species which are later utilized for signal amplification. We have genetically fused the antibody binding domain with APEX2 (APEX2-ABD) to give dual functions, antibody binding and peroxidase activity, simultaneously. APEX2-ABD complex is successfully overexpressed and purified with chromatography and characterized with mass spectrometry. Broad binding capability of APEX2-ABD fusion protein to various types of antibodies originated from rabbit, rat, and mouse is confirmed with quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) analyses and the peroxidase activity of APEX2-ABD fusion protein is verified with Amplex red assay. Finally, we apply APEX2-ABD complex as a substitute of conventional HRP-conjugated secondary antibody in the tyramide signal amplification (TSA) detection and we observe significant signal enhancement in cell and tissue imagings.

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Chapter 1. Introduction

1.1 Enzyme-based Assay System

Enzyme-based assay systems employ the inherent properties of the enzymes that could selectively recognize and specifically bind the target substrates, allowing to be readily detected with significant catalytically activity of the enzymes. To aim this, a number of scientists have reported novel enzymes and developed a wide variety of enzyme-based assays which could be practically used to colorimetric[1] or fluorometric[2] detection and signal amplification[3]. Some examples of commonly used enzymes are esterase that hydrolyzes ester-compound into alcohol and acid forms[4] and tryptophanase that catalyzes L-tryptophan to indole, triggering very intense odor, easily detected by even human nose[5].

One of the most universally used enzymes is peroxidase, which catalyzes reduction of hydrogen peroxide and also oxidizes various target substrates. Among them, horseradish peroxidase (HRP) is the most widely applied enzyme in many different types of applications such as spectrophotometry, fluorometry and electro-analytical chemistry, due to its ability to generate singlet oxygen, significantly affecting surrounding molecules[6-8]. In particular, the HRP converts chromogenic substrates, including o-phenylenediamine (OPD) and 3,3'-diaminobenzidine (DAB) to molecules having colors and also catalyzes chemiluminescent substrates (Enhanced chemiluminescence (ELC)), producing the light. Therefore, the HRP has been utilized as a labeling agent to chemically conjugate with biomolecules. For example, the immunoglobulins can be served as templates to conjugate with the HRP in order to visualize antigen–antibody interaction by catalyzing the substrate in hydrogen peroxide-dependent manner. Typically, HRP-conjugated primary and secondary antibodies are tremendously used in HRP-based detection methods for immunochemistry applications like immunoblotting, immunohistochemistry and enzyme-linked immunosorbent assay[1] (Figure 1-1).

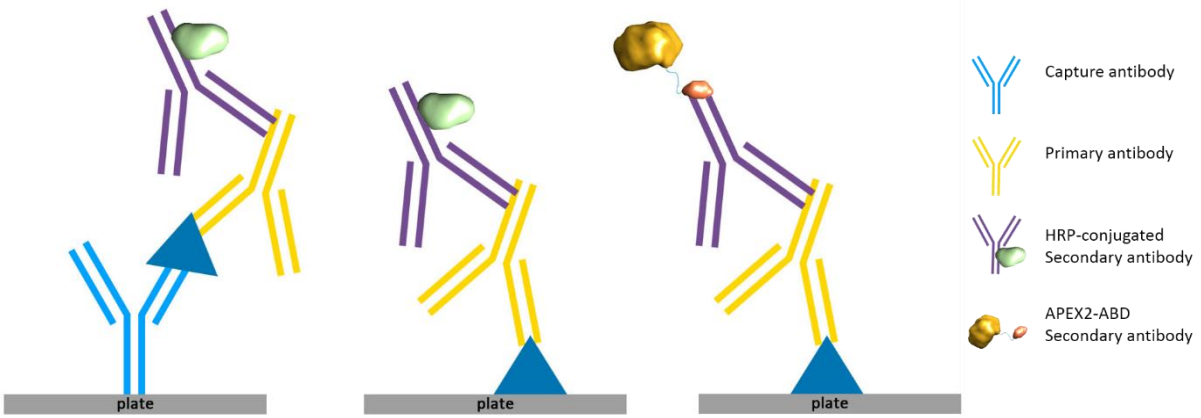


Figure 1-1. Schematic representation of detection system applied to protein-protein interaction.

The significant changes caused by the HRP can be detected by various techniques, including spectrophotometric detection, because the HRP-based immunoassay system is amenable to handle using simple plates because of its relatively high sensitivity with the combinations of selective affinity that the antibodies possess. It is important to detect the specific interaction between antigen and antibody, especially for early diagnoses and prognoses of the target diseases in analytical tools[9, 10]. To modify the antibodies with the HRP or other relevant molecules like biotins, the minor parts of the antibodies need to be used without damaging overall structure and functions of the antibodies. However, it is very difficult to precisely handle and control those various chemical conjugations because the antibodies have complex structures, sometimes causing inactivation and unstable and random orientation of the antibodies[11, 12]. In addition, conjugation of HRP with antibodies has very low reproducibility and even quantification of the number of conjugated HRP molecules is hardly defined.

1.2 Targeted Delivery Modules

The proteins that have high affinity to specific targets are very promising to be used in various applications. Among them, antibodies have been considered as targeting ligands because of their high affinity and selectivity to their targets[13]. Moreover, a number of antibodies to various target molecules have been developed to be available in commercial. However, as mentioned above, it is very challenging to use directly cross-linking strategy to conjugate antibodies with other functional molecules, resulting in the structure-disordering and malfunction. These chemical conjugations and related approaches could even cause impair the binding capability of antibodies due to changes in their antigen binding sites and denaturation of the antibodies.

The IgG molecule, the common antibody type, consists of a large multi-domain protein and use only a small part of the antibody to recognize their complement antigens. Thus, it is possible to develop the novel protein structure in terms of small size and orientation as an alternative of the whole antibody. Recently, many studies to configurate novel affinity proteins have been robustly reported for therapeutic and imaging agents and further biotechnological applications Nanobody or Affibody. The general applications of using Affibody are illustrated[14] (Figure 1-2).

Going beyond, Antibody-binding proteins, such as protein A, G, and L, can capture the Fc region of immunoglobulin by non-covalent protein-protein interaction[15, 16]. These are derived from the cell wall of bacteria, which has an immune evasion strategy to neutralize humoral immunity of host. One of the antibody-binding domains (ABD), Z domain isolated from protein A[17], which is comprised of five homologous Ig-binding domains, will be used as targeting moiety in this paper.

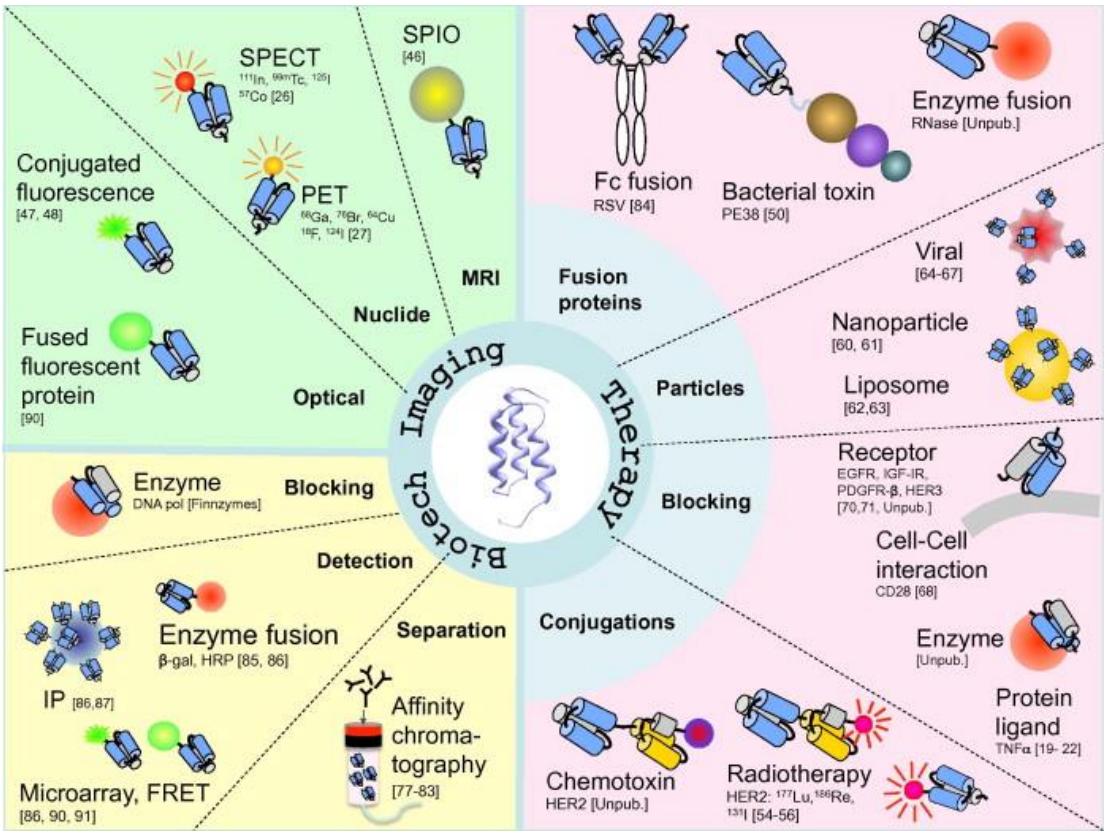


Figure 1-2. Illustration map of application using Affibody molecules. Applications are divided into three research areas: imaging, therapy and biotechnology[14].

1.3 Signal amplification system

To detect the target molecule easily, the population of target molecules per unit sample volume is substantial in all biological detection methods. Therefore, many researches have long investigated to detect molecules at their intrinsic levels, which can be greatly improved by many orders of their magnitude. Also, molecular targets within the cells have spatial and temporal variations that could signal amplification systems.

There are diverse signal amplification strategies that could detect even very low population of target molecules. One of the most well-known and well-studied methods is the way to use enzyme, which turns over fluorogenic or chromogenic substrates. The enzymatic reaction results in target-associated signal enhancements using specific dye-conjugated with reagents that have strong affinity to targets. The most broadly recruited enzymes for this assay is horseradish peroxidase (HRP).

As one typical type of those signal amplification methods, the tyramide signal amplification (TSA) assay has lately been reported with its huge potential in that the TSA assay significantly enhances their sensitivity for detection. The reaction for this assay tends to involve biotin- or fluorophore-conjugated tyramide, which can act as a substrate for peroxidase. As a result, the peroxidase catalyzes phenolic group of the tyramide using hydrogen peroxide, leading to generate reactive tyramide radicals that induce covalent bond.[18]

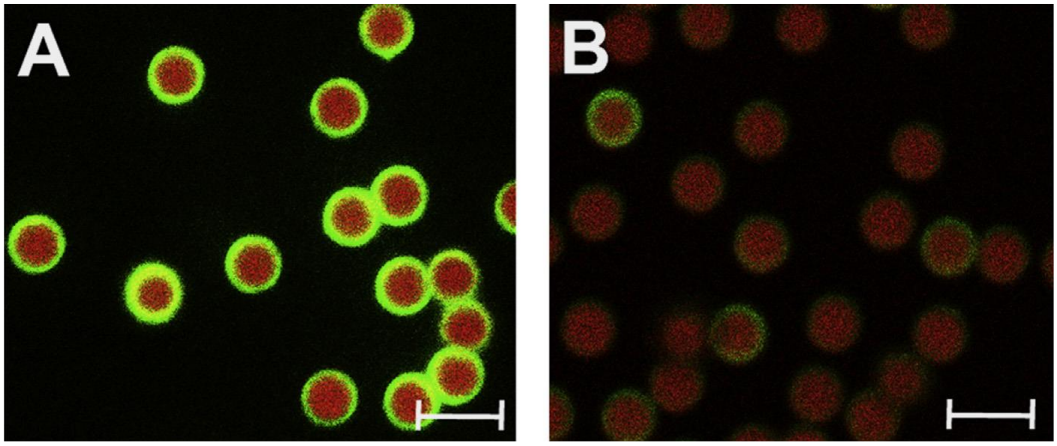


Figure 1-3. Enhancement of fluorescent signal by tyramide signal amplification (TSA). By indirect immunoassay, signal enhancement was performed to detect biotinylated IgG-coupled magnetic beads, which were incubated with primary antibodies (mouse anti-biotin IgG) and secondary antibodies, goat anti-mouse IgG conjugated with either (A) HRP (1:20,000) or (B) FITC (1:100). The signal amplification with HRP activity was confirmed using a 10 min amplification of TSA 488.[19]

1.4 Research Outline

In this thesis, a novel monomeric fusion protein that has enzyme activity and antibody binding domain simultaneously that could be utilized in target-specific signal amplification assay. We would like to select a new type of peroxidase as an alternative form of the horseradish peroxidase (HRP), because the HRP is a relatively large 44 kDa glycoprotein which can be only produced using complicated methods, although the HRP has powerful catalytic activity with high sensitivity. In detailed, the HRP becomes inactive when expressed in the mammalian cytosol, presumably because its four structurally essential disulfide bonds do not form in reducing environments. Although the HRP was re-engineered for enhanced activity in the cytosol to overcome the limitations by removing its disulfide bonds, a noticeable research has been reported for peroxidases that are naturally active in reducing environments.

(A) HRP (B) APEX

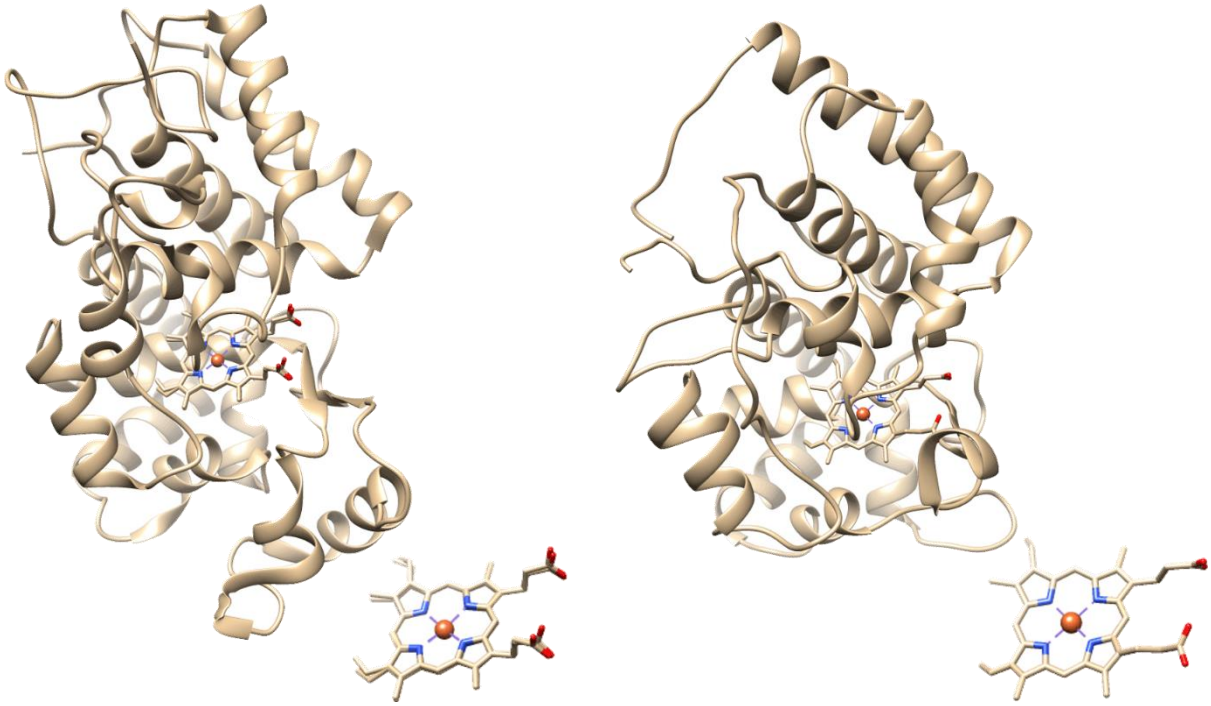


Figure 1-4. Heme-containing peroxidase enzymes which catalyze the H2O2-dependent oxidation of a variety of substrates, horseradish peroxidase (A) and ascorbate peroxidase (B).

To overcome this limitation, we chose ascorbate peroxidase (APX), which is a 28 kDa class I cytosolic plant peroxidase that lacks disulfide bonds[20, 21]. The wild type APX (WT APX) forms a problematic homodimer, which can disturb a protein's natural function. Martell's group previously engineered WT APX into monomeric APX (Engineered ascorbate peroxidase, APEX) which has enhanced activity and stability through three times of point mutations[22]. In addition, Lam's group suggested a mutant which shows enhanced catalytic activity by site-directed mutagenesis from alanine 134 to proline ($APEX^{A134P}$, APEX2)[23]. This APEX2 protein can be easily and largely produced using overexpression system of *E.coli*, compared to that of the HRP.

To impart targeting moiety, the Z domain from protein A was utilized as an antibody binding domain (ABD) because of its specific affinity and versatility to bind fragment crystallizable (Fc) region of the various antibodies originated from mouse, rabbit, rat, and even human. This is advantageous over the HRP-conjugated antibody in that the antibody requires a specific pair to make a complex between primary and secondary antibody in terms of the antibody's origin and the precise control for conjugation is very hard such as the stoichiometry.

(A) (B)

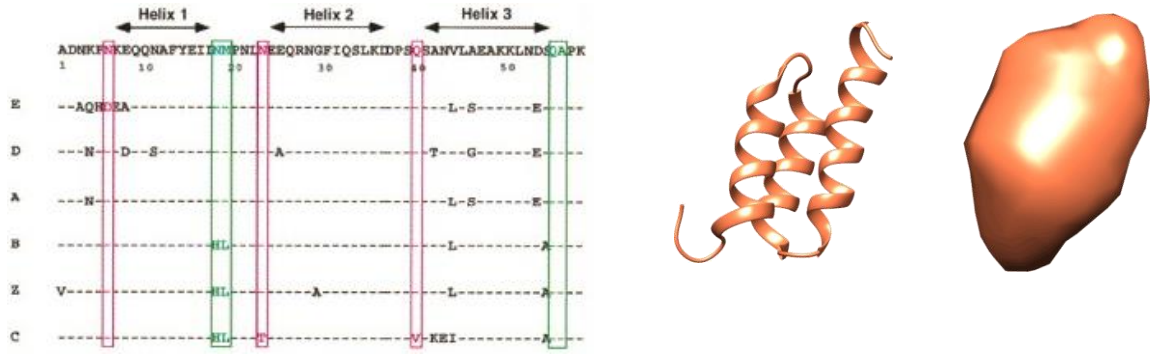


Figure 1-5. (A) Sequence alignment of the IgG-binding domains of staphylococcal protein A[17]. (B) The crystal structure of the Z domain (PDB ID : 2SPZ) from staphylococcal protein A.

Until now, APEX was mainly used in proteomic mapping of endogenous mitochondrial proteins or endoplasmic reticulum membrane proteins[24, 25]. This project, however, was proceeded through quite a different approach. In order to impart both peroxidase activity and targeting moiety to one molecule at the same time, we combined those two proteins via genetic modifications and engineered the novel fusion protein between APEX2 and ABD so as to possess target-specific enzymatic activity that could be used in signal amplification assay.

Chapter 2. An Enzyme-mediated Target-specific Signal Amplifier

2.1 Summary

APEX2 is monomeric form which has improved catalytic activity. It is known that APEX catalyzes hydrogen peroxide, generating reactive oxygen species, which promote fluorogenic or colorimetric substrate turnover. Here, we developed the APEX2-ABD fusion protein which has Ig-binding capability as well as catalytic activity that could be further applied to signal amplification assay.

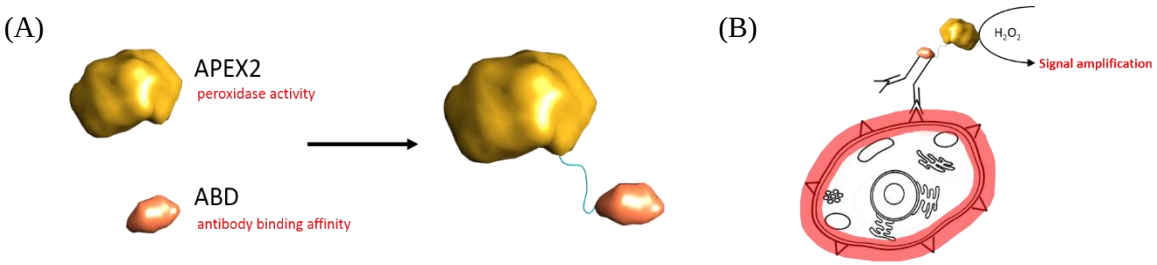


Figure 2-1. (A) Developing fusion protein by genetic insertion method. (B) Schematic image of signal amplification using APEX2-ABD fusion protein.

2.2 Materials and Methods

2.2.1 Antibody-binding Domain insertion and Fusion Protein purification

The Z domain (58 amino acid) is inserted into C-terminus of APEX2 with 27 amino acid linker and subcloned into the pTRC99A bacterial expression vector from pETduet vector. Recombinant DNA is transformed into competent Escherichia coli strain BL21 (DE3). One colony was amplified in 5 mL Luria Broth and subsequently seeded into 1L LB media. APEX2-ABD is overexpressed overnight at 30 ℃ after adding 420 uM isopropyl p-D-1-thiogalactopyranoside (IPTG). The bacterial cells were pelleted from media by centrifugation and resuspended in 30 mL of lysis buffer (50 mM sodium phosphate and 100 mM sodium chloride, pH 6.5). For catalyzing the hydrolysis of bacterial cell wall, the suspension was treated by lysozyme (50 ug/ml) and incubated for 30 min at 4 ℃. The solution was sonicated for 10 min in 30 s intervals, followed by centrifugation at 12000 g for 1h at 4 ℃. The supernatant was further purified by using an immobilized metal affinity chromatography (IMAC) using the hexa-histidine tag of the N-terminus region of APEX2-ABD. Filtered extract was loaded into 1 mL Ni-NTA agarose affinity column HisTrap™ (GE healthcare, code number 17-5319-01) and eluted by a linear gradient from 5 to 100 % of 1M imidazole elution buffer (20 mM sodium phosphate, 500 mM sodium chloride and 1M imidazole pH 7.4). The free imidazole was washed out by dialysis with buffer

(50 mM sodium phosphate and 100 mM sodium chloride, pH 6.5) and the purified APEX2-ABD is characterized by UV/visible spectroscopy and SDS-PAGE.

2.2.2 Quartz crystal microbalance (QCM) measurements

To check binding affinity to immunoglobulin, Q-Sense E4 and Standard gold QCM sensors (Q-Sense, Sweden) is used as described previous study[13, 26]. The system condition was in flow mode with a pump and temperature was maintained at 25.0 ± 0.1 ℃. First, system was kept an equilibrium by phosphate buffer (100 mM NaCl and 50 mM sodium phosphate, pH 7.4), and consequently 1 mg/ml APEX2 or APEX2-ABD solution was treated. After maintaining the signal balance using buffer, 0.2 mg/ml rabbit or mouse IgG in phosphate buffer were introduced. The QCM chips were washed by phosphate buffer at each intervals.

2.2.3 Surface plasmon resonance (SPR) analysis

SPR experiments were performed with CM-5 gold chips on a Biacore 3000 device at 25 ℃ using a filtered PBS buffer as a running solution. CM-5 sensor chip is coated with carboxymethylated dextran to which molecules can be coupled with a Rabbit or mouse IgG to the surface of a sensor chip by standard amine-coupling chemistry[27]. For pre-concentration of the sensor chip, 0.1 mg/mL of rabbit or mouse IgG was injected for a short time and subsequently 60 uL of a 1:1 mixture of EDC (0.5 mg/mL) and NHS (0.5 mg/mL) was infused onto the chip at a flow rate of 10 μL/min to activate carboxyl groups on the dextran surface. 0.1 mg/mL of rabbit or mouse IgG was added (volume was determined by desired RU). Excess reactive carboxylated groups were blocked with 1 M ethanolamine (pH 8.0). As APEX2-ABD flowed during about 3 min at every concentration, the binding domain of APEX2-ABD was captured by rabbit or mouse IgG coupled into CM-5 chip a various range of sample concentration.

2.2.4 Cell culture

Before fluorescence confocal imaging, APEX2-ABD had to be labeled with 10 mol equivalents of fluorescein-5-maleimide (F5M) at room temperature with vigorous shaking overnight. For removing free dye, fAPEX2-ABD was dialyzed against PBS overnight.

SK-BR-3 was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum, 1% streptomycin, and 25mM HEPES and 25mM NaHCO3 in a humidified atmosphere of 5% CO2 and 95% air at 37℃. SCC-7 cells were cultured in RPMI1640 supplemented with 10% FBS and 1% penicillin-streptomycin. SK-BR-3 and SCC-7 cells (8×10^4/well) were grown in a 12-well culture plate (SPL, 30012) by attaching on microscope cover glass (18mm Φ). The cells were fixed with

4% paraformaldehyde in PBS for 20 min and washed three times with PBS containing 0.1% Tween-20. This process is followed by blocking to prevent APEX2-ABD binding to background with blocking reagent (5% BSA, 5% FBS, and 0.5% Tween-20 in PBS) at 4℃ for over 12h. After blocking buffer was removed, 140 nM anti-rabbit Erb2 antibody (Abcam) or anti-mouse CD44 antibody (Biolegend) is respectively treated to the plate and incubated at 4℃ for 1h, whereas negative control was added as same concentration of blocking reagent. The free antibodies was washed three times with PBS containing 0.1% Tween-20 and subsequently 980 nM fAPEX2-ABD sample was added to each well and incubated at 4℃ for 1h. Washing process was needed as previous step. Before sealing, sample-treated cells were stained with 4', 6-diamidino-2-phenylindole (DAPI). Fluorescence images are obtained using an Olympus Fluoview FV1000 (UOBC) confocal microscope.

2.2.5 Fluorescence intensity measurements

To verify peroxidase activity of APEX2-ABD, plate-level assay was preceded using Amplex Red® reagent (Molecular probe®, cat# A12222) which is converted into red fluorescent product, resorufin, when it is oxidized by hydrogen peroxide[28-31]. There were two different experimental strategy; enzyme concentration-dependent and substrate concentration-dependent ways with flow of time. Fluorescence intensity was checked by fluorescence microplate reader (Tecan Group Ltd., Infinite® 200) which has 70000 value detection limit of fluorescence intensity. Firstly, 100 uL of 11 range of APEX2-ABD samples were pipetted to 96 well plate and subsequently added 100 uL of a reaction solution (50 uM Amplex Red® and 50 uM H2O2) to each plate. Secondly, reaction solution was two-fold diluted 10 times and loaded to 96 well plate. 100 uL of 1 uM APEX2-ABD (final concentration was 500 pM) was plated and detected by microplate reader. All data were acquired from three repeated experiments.

2.2.6 Tyramide Signal Amplification assay

Cell culture has been performed as above. In this case, quenching process was needed to block endogenous peroxidase activity by 1-3 % H2O2 in PBS before the blocking step. Primary antibody labeling was performed for 1 hour at room temperature and the cells were washed three times with PBS containing 0.1 % Tween-20. Subsequently, fAPEX2-ABD was treated during 1 hour at room temperature and free samples were rinsed three times with wash buffer. Finally, the cells were added to tyramide working solution (tyramide stock and 0.0015% H2O2 in amplification buffer) and incubated for 15 min at room temperature. Since the signal of this method was too high, samples had to be surely washed.

2.3 Results and Discussion

2.3.1 Antibody-binding Domain insertion and Fusion Protein purification

To construct fusion protein which has both catalytic peroxidase activity and Ig binding affinity that could bind to the antibodies, we prepared a gene encoding the APEX2, which is followed by subcloning with ABD into pTRC99A expression vector (Figure 2-2), which gave a lower yield of proteins but a high percentage of heme incorporation[22]. The 27 amino acid linker (WNSGGGLVAR GSGGGCGGGTGGGSGGG) provides conformational flexibility and reduces steric hindrance between two functional domains[32].

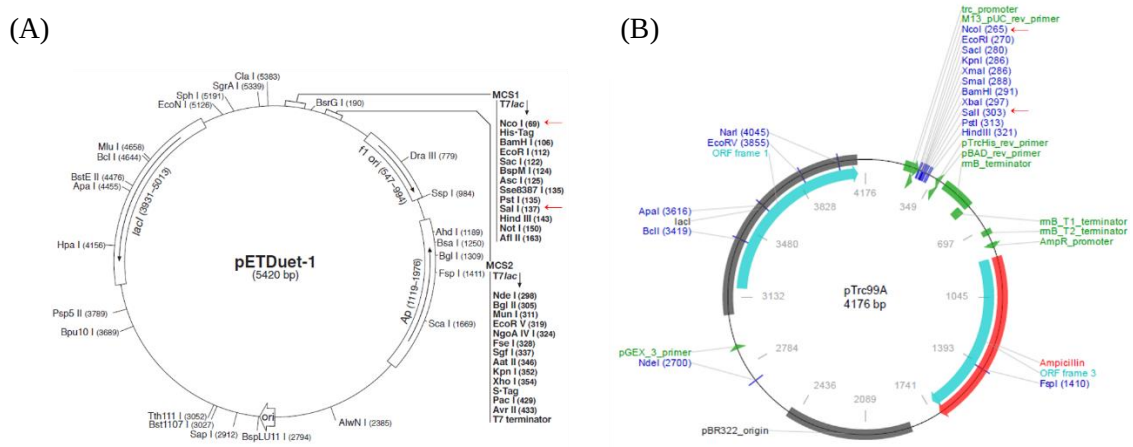
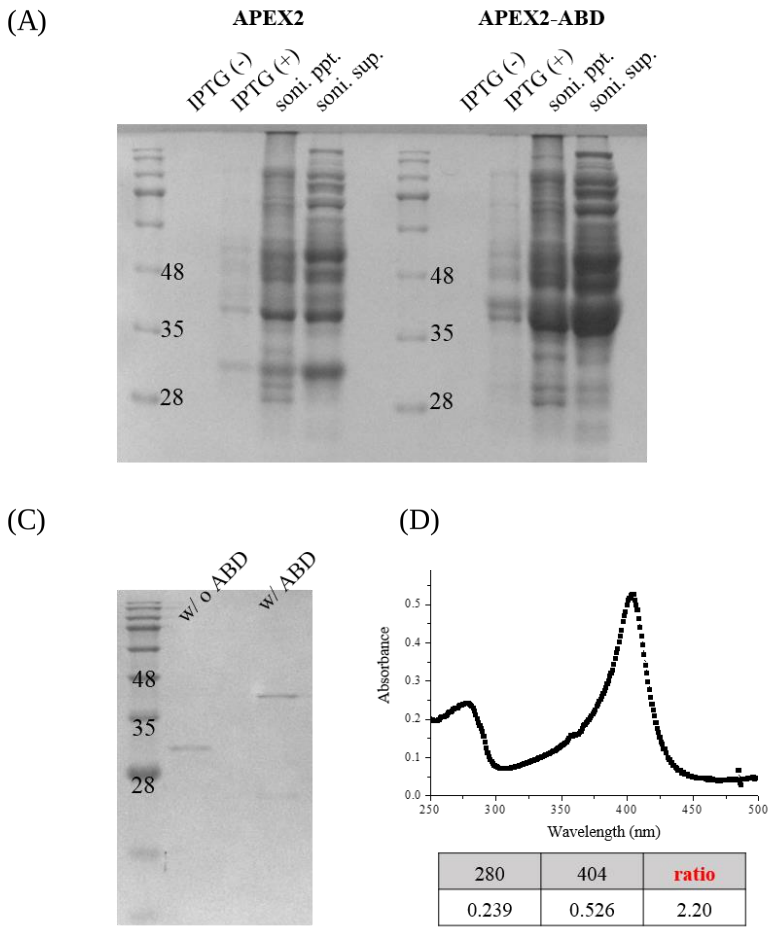
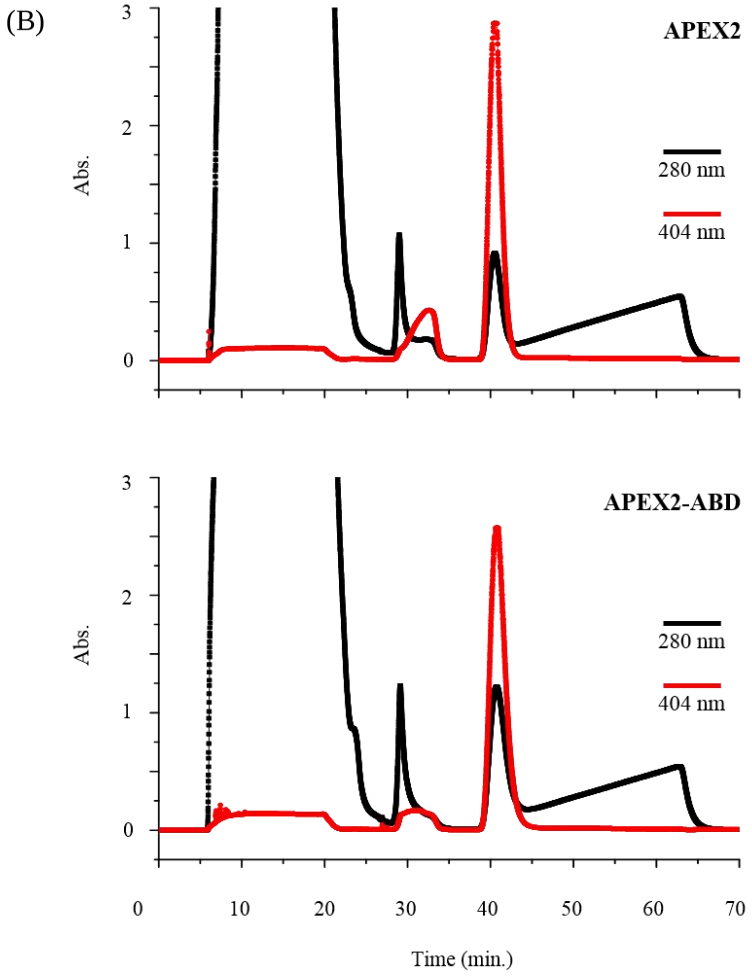


Figure 2-2. Two types of expression vector. One is pETDuet which make a high yield of proteins but a low percentage of heme incorporations (A), the other is pTrc99A giving a high percentage of heme (B).

The APEX2 and the APEX2-ABD fusion protein (APEX2-ABD) were overexpressed in *E. coli* and we finally found out the most optimal culture conditions (30℃, overnight culture) through a series of experiments. Thus, we could obtain a soluble protein even after sonication process (Figure 2-3A) and further purified by immobilized-metal affinity chromatography (Figure 2-3B), because its hexa-histidine tags of APEX2-ABD provide binding affinity to Ni-NTA columns. Purified fusion protein is characterized by SDS-PAGE and ultraviolet–visible (UV/vis) spectroscopy (Figure 2-3D). The molecular weights of proteins, the APEX2 and APEX2-ABD, were identified by SDS-PAGE (Figure 2-3C) in accordance with calculated value 29557.1 Da and 38297.6 Da respectively, which indicates the successful fusion of the ABD protein to APEX2 protein. Since the catalytic activity of the APEX2 is directly involved with the amount of heme incorporation. In detailed, as the amount of the heme is increased, the peroxidase activity of APEX2 is significantly enhanced. Therefore, we examined the ratio of the amount of heme cofactor to that of protein using UV/vis spectroscopy. Therefore, the proportion of heme cofactor (at 404 nm) to total protein (at 280 nm) can be an important factor (Figure 2-3D, bottom) whose value is 2.2. We found out optimum culture condition (30℃, overnight culture) through a series of experiments.

Figure 2-3. Characterization of APEX2 and APEX2-ABD. (A) SDS-PAGE analysis to show the solubility of the APEX2 and APEX2-ABD. (B) IMAC profiles of the APEX2 and APEX2-ABD with 5-100% gradient of 1M imidazole. (C) SDS-PAGE analysis indicates the molecular size of the APEX2 and APEX2-ABD. (D) UV/vis spectra of the APEX2-ABD (top) to show successful incorporation of heme (A404) and the calculated ratio value of A404 to A280 (bottom).





2.3.2 Antibody-binding Domain

To verify the Ig binding affinity of the Z domain of APEX2-ABD, we used quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) in order to easily detect bimolecular interaction as a label-free and real-time monitoring.

Firstly, the QCM experiment is depending on deposition of molecules on sensors, representing sensitivity to the masses of deposited molecules. As the APEX2 w/ or w/o ABD deposited on the QCM sensor, resonance frequency was decreased and maintained its value until saturation point to completely cover the sensor chip surface with sample. Washing procedure was needed to remove any free or loosely bound proteins. Subsequently, we introduced rabbit, mouse and rat IgG solution over the monolayered QCM sensor and the frequency was drastically decreased, whereas there was no change in case of APEX2 only protein (Figure 2-4). Therefore, this result indicates that the APEX2-ABD has specific binding ability to various forms of the antibodies, resulting in versatile use of the APEX2-ABD without thinking any pairs to conjugate.

Secondly, SPR analysis is selected to further investigate binding affinity between APEX2-ABD and various types of IgGs, In contrast to the QCM studies, we first immobilized three kinds of polyclonal IgGs originated from rabbit, mouse and rat on the surface of a carboxylated-dextran surface coupled to a thin gold surface (SPR CM-5 sensor chip) through chemical modification with the amine coupling through EDC/NHS reaction. 1M ethanolamine is injected to prevent additional binding between residual reactive amine groups and following sample. Then, we introduced the APEX2-ABD and APEX2 with several different concentrations. Each experiment was performed after regeneration of the surface by base-washing and subsequent equilibration with the proper binding buffer. A significant gradual increase in SPR responses (RU) were observed upon introduction of the APEX2-ABD at various concentrations while no apparent change of RU was observed upon introduction of the only APEX2 (Figure 2-5, top). These data suggest that the ABD of the APEX2-ABD may act to cooperatively capture a variety of IgGs maintaining APEX2-ABD relatively tightly complexed with the target antibody.

According to the binding kinetics of the APEX2-ABD from SPR analyses, we obtained kinetic data (Figure 2-5, bottom) such as dissociation constant values. The response signal from the SPR detector is proportional to the ratio of the mass of protein to the surface area, which means the SPR response units (RU) were increased to concentration-dependent manner. Although the dissociation constant was micromolar unit according to 1:1 Langmuir binding model, we confirmed the interaction through *in vitro* cell targeting imaging strategy and demonstrated potential to be used in targeted delivery applications. We presumed that the low level of binding affinity was possibly due to the fusion of the APEX2 proteins, which could change the flexibility of the binding affinity of the ABD.

Figure 2-4. QCM profiles of both the APEX2 (black lines) and the APEX2-ABD (red lines). All three types of antibodies were used; rabbit (left), mouse (center) and rat (right).

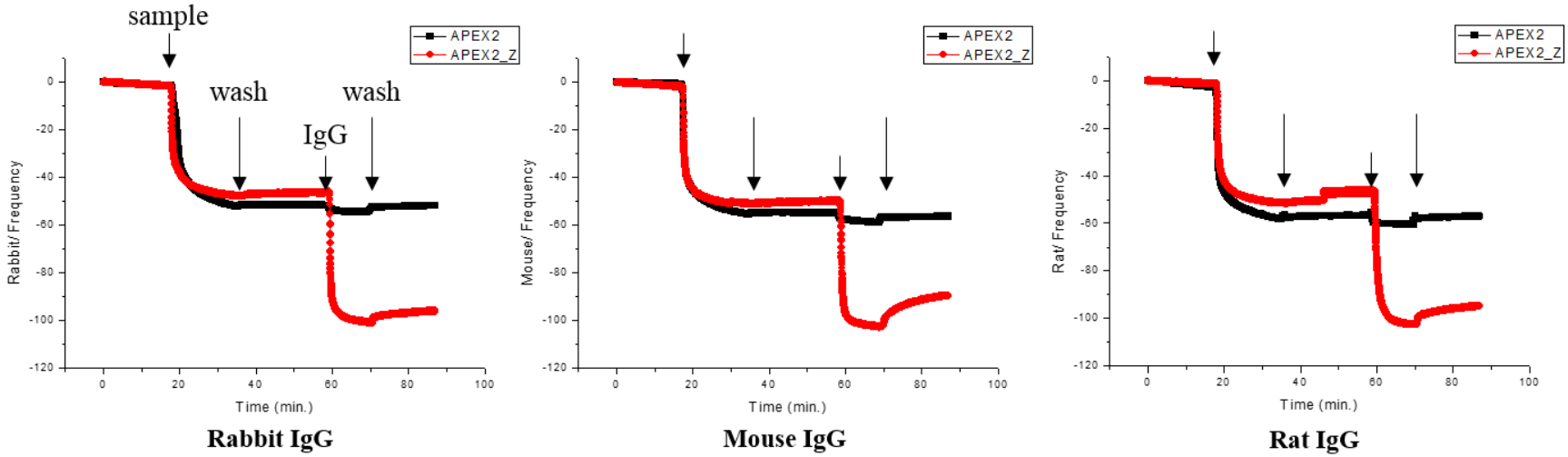
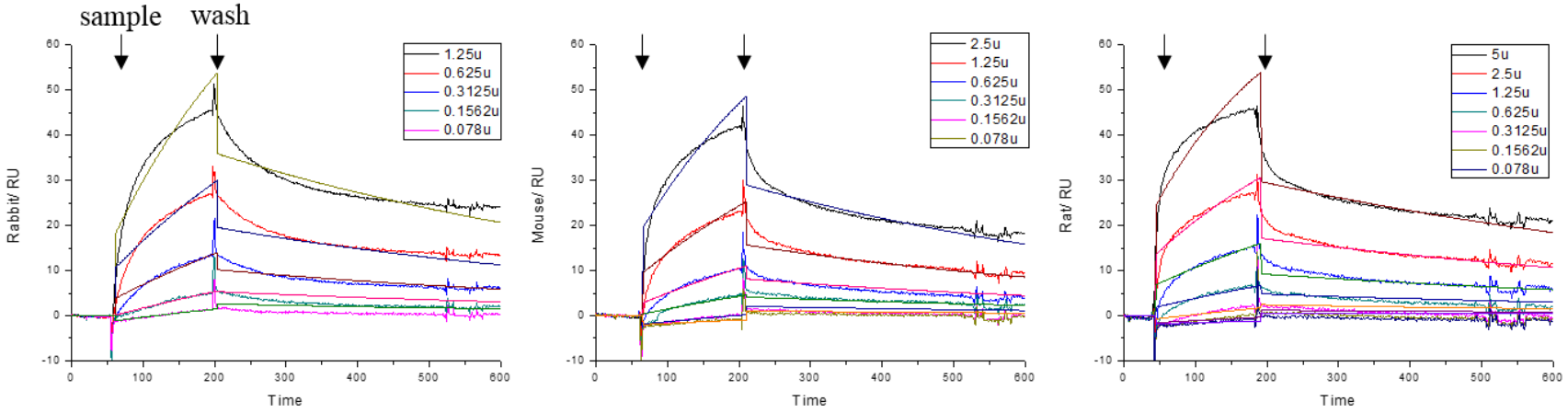
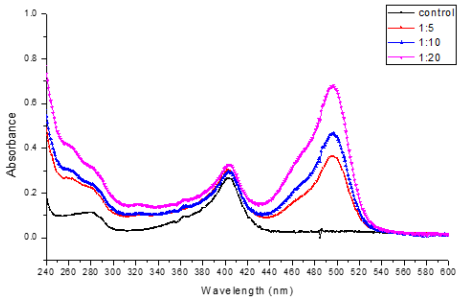


Figure 2-5. SPR profiles of the APEX2-ABD with a serial 1/2 dilution. All three types of antibodies were used; rabbit (left), mouse (center) and rat (right). The fitted lines corresponding to the measured lines are 1:1 Langmuir binding fitting models upon concentration gradients (bottom, table).

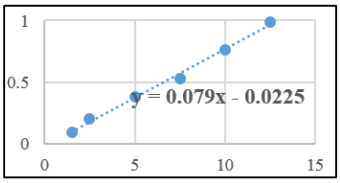


Antibody	k_{On}(s)	k_{OFF}(1/s)	K_A(1/M)	K_D(M)	Chi2
Rabbit	2.05E3	1.39E-3	1.47E6	6.79E-7	1.92
Mouse	939	1.55E-3	6.06E5	1.65E-6	0.764
Rat	882	1.17E-3	7.54E5	1.33E-6	1.42

Before evaluating targeted delivery of the APEX2-ABD, the APEX2-ABD was chemically conjugated to possess inherent fluorescence. We labeled 2 cysteine residues of APEX2-ABD fusion protein with fluorescein-5-maleimide (fAPEX2-ABD) to investigate the targeted binding of antibody-presented fAPEX2-ABD toward each specific cells (Figure 2-6).



standard curve	1.5 uM	0.088626
	2.5 uM	0.200562
	5 uM	0.381274
	7.5 uM	0.527134
	10 uM	0.757594
	12.5 uM	0.991283



sample	control	0.0262	0	-	F5M(uM)	RATIO
	1:05	0.3568	0.33053	4.468734	44.68	0.980
	1:10	0.4582	0.43193	5.752278	57.52	1.262
	1:20	0.6692	0.64293	8.423165	84.25	1.848

Figure 2-6. Quantification the number of fluorescein-labeled cysteine residue by standard plot using UV/visible spectra.

To examine whether the APEX2-ABD can make a complex with various antibodies in spite of its low binding affinity according to the SPR analysis and also those complexes can selectively bind its target cells at the cellular level, we prepared target cell lines and selected antibodies corresponding to their target cell lines. We chose anti-Erb2 antibody (rabbit), anti-CD44 antibody (mouse and rat) to show versatile binding regardless of the origin of the antibodies. Each antibody targets SK-BR-3 and SCC-7 respectively. The Fluorescence images (Figure 2-7) indicate that fAPEX2-ABD cooperatively and tightly interacts with the surface marker of each specific cancer cells, while negative control (fAPEX2-ABD w/o antibodies) cannot get a chance to reach them. This represents that the Z domain has proper binding affinity with the Fc region of various polyclonal IgGs.

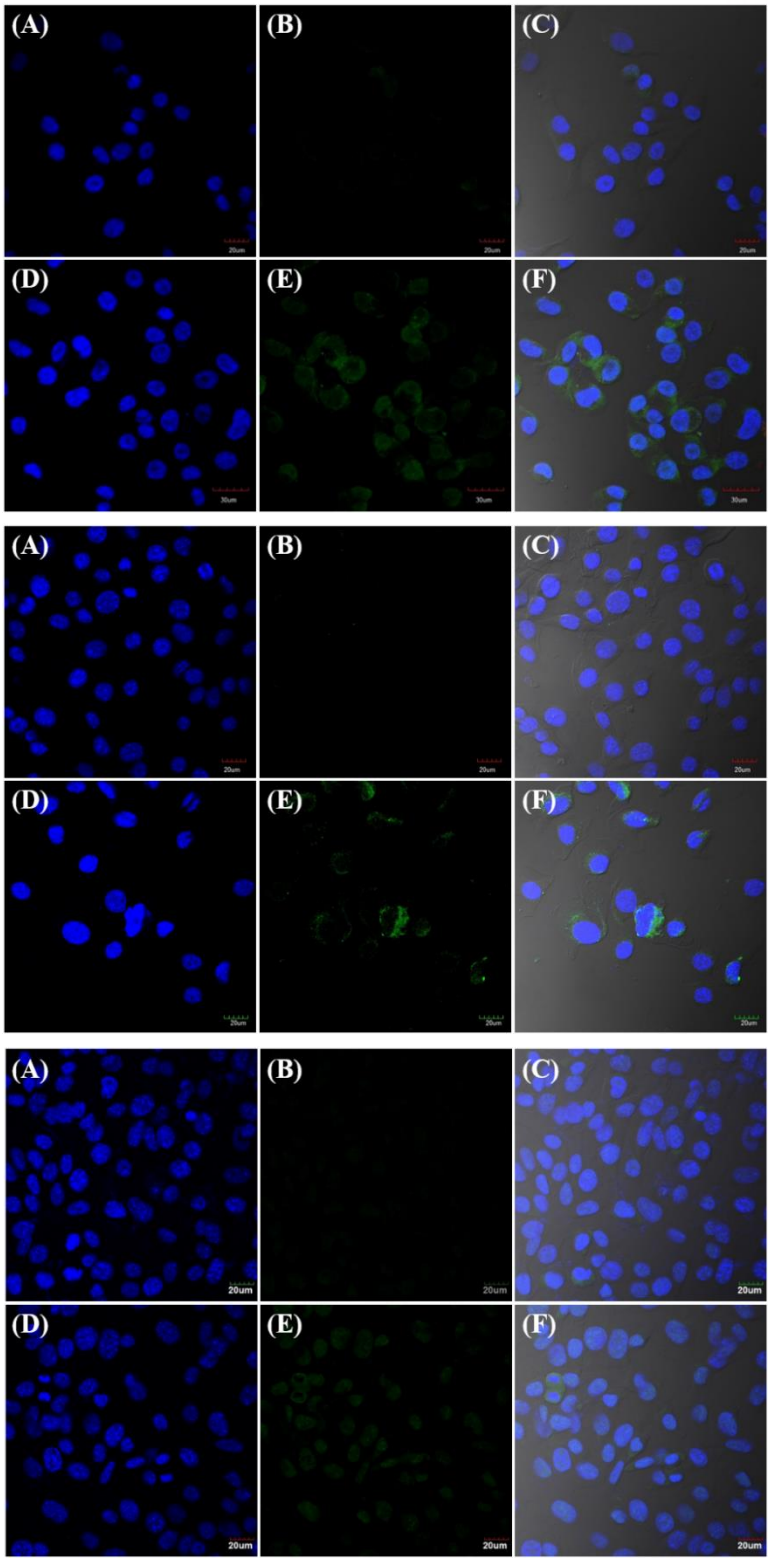


Figure 2-7. Fluorescent microscopic images of SKBR3 (top, rabbit IgG) and SCC-7 cells (center, mouse IgG and bottom, rat IgG) treated with Ab/fAPEX2-ABD and APEX2-ABD. DAPI (left), fluorescein (middle) and merged (right) images were presented. *(A)-(C) and (D)-(F) represent negative control and APEX2-ABD, respectively. The nuclei were visualized as blue.

2.3.3 Catalytic activity test

Firstly, reaction profile was obtained depending on the concentration of APEX2-ABD. The fluorescence intensity changes with time were recorded after adding different concentrations of APEX2-ABD when concentration of substrate Amplex Red® was 25 uM. As shown in Figure 2-8 (left panel), fluorescence intensity increased with flow of time. The kinetics of resorufin production was observed to be faster with higher concentration of APEX2-ABD. The fluorescence intensity versus the concentration of APEX2-ABD at the 10 min time point was plotted in Figure 2-8 (right panel). It was clearly observed that the fluorescence value increased gradually with the increasing concentration of APEX2-ABD. The detection limit was considered as 15 to 31 pM. Although the fluorescence intensity exceeded the detection range of the microplate reader, the fluorescence intensity had a linear relationship with the reaction time in the range from 15 to 500 pM (Figure 2-8, right panel).

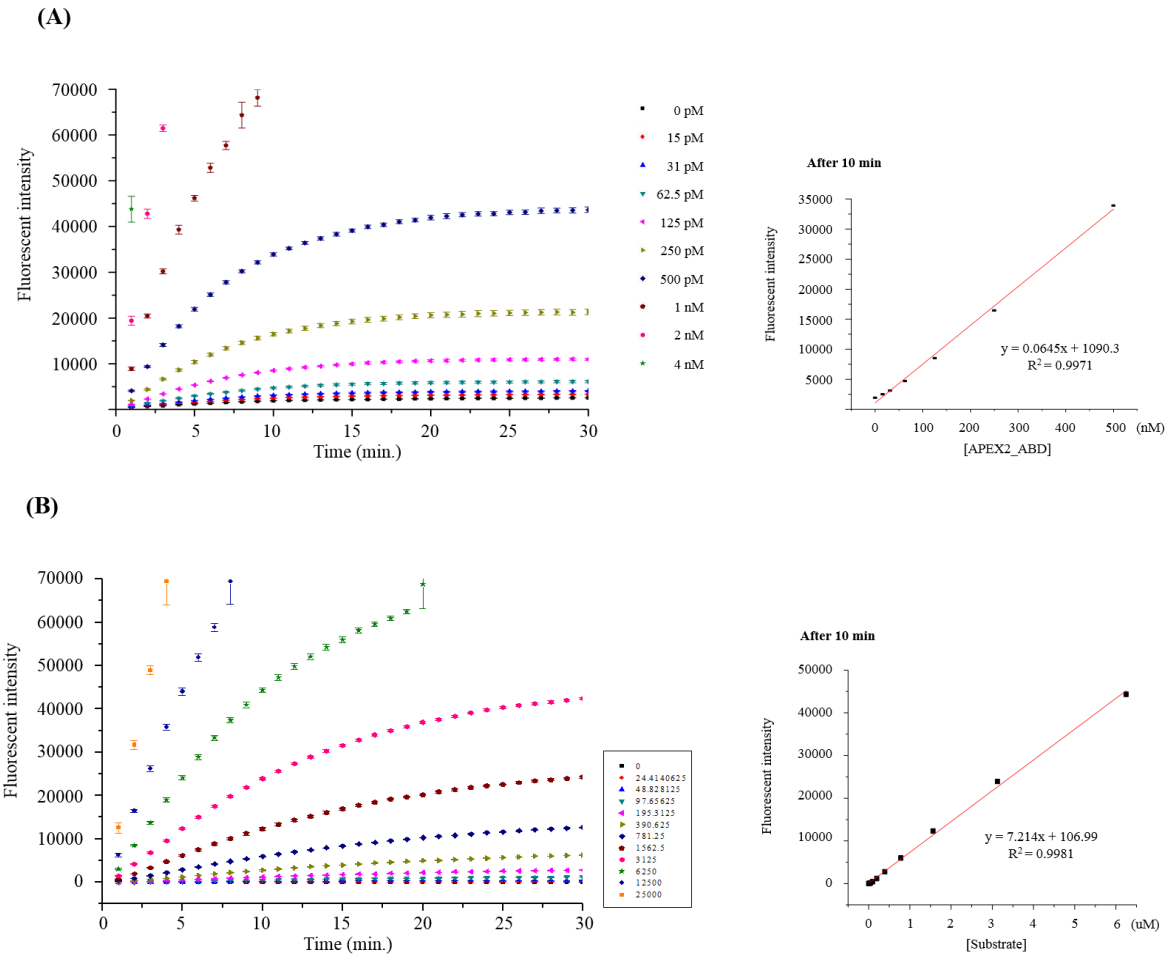


Figure 2-8. The fluorescence intensity profiles with concentration of enzyme-dependent (A) and concentration of substrate-dependent (B) with time flow 30 min (left panels). Right panels shows linearity plot during 10 min.

2.3.4 Tyramide Signal Amplification kit

Finally, we apply the novel APEX2-ABD to enzyme labeling by using tyramide signal amplification (TSA) assay. The dye-labeled tyramide derivatives is employed as target-specific affinity reagent, catalyzed by the use of peroxidase. The deposition of activated dye-labeled tyramide conjugate on the enzyme site results in localized enhancement of fluorescent signal. While HRP-conjugated secondary antibodies require the precise host pair to interact with primary antibodies, APEX2-ABD can be utilized to detect various types of antibodies originated from rabbit, mouse and rat *in vitro* cell level study (Figure 2-7).

For TSA assay, each cell was treated with the fAPEX2-ABD, followed by primary antibodies, then the mixture of Alexa555-conjugated tyramide and 0.0015% H_2O_2 were incubated for 10 min. The fAPEX2-ABD was targeted appropriately to the target cells and strong signal amplification provided by the TSA method was observed (Figure 2-9, red filter image), representing signal on-off system.

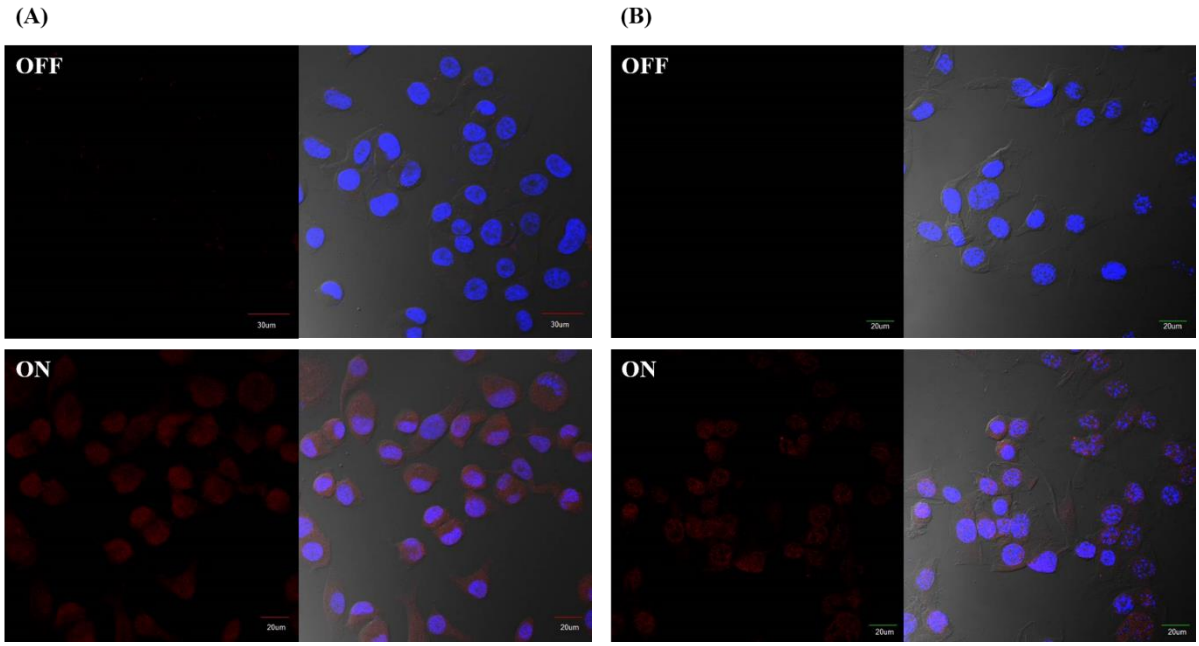
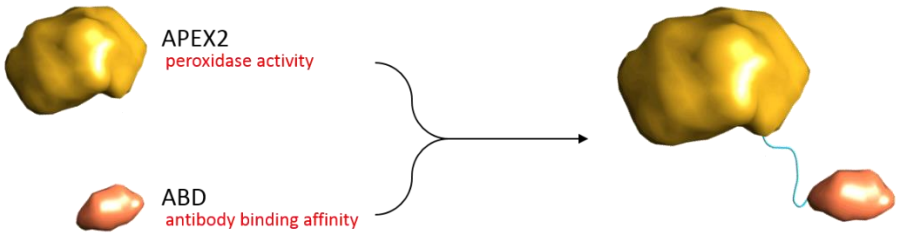


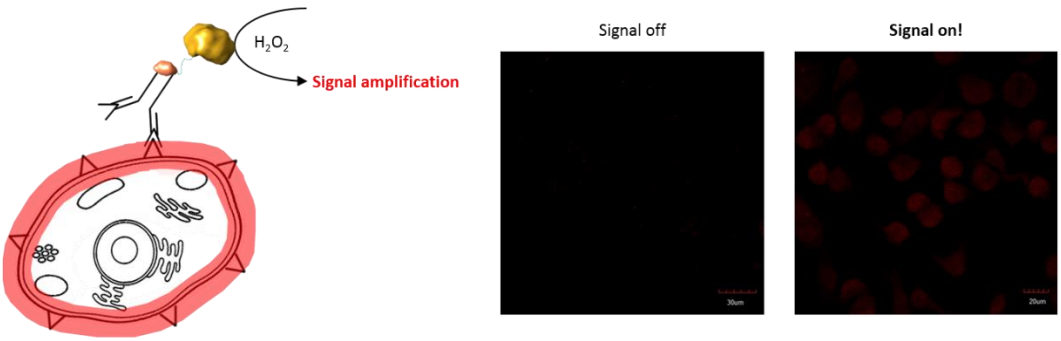
Figure 2-9. Fluorescent microscopic images of SKBR3 (A) and SCC-7 cells (B) treated with the APEX2-ABD only (upper line) or APEX2-ABD complexed with the target antibodies (bottom) and subsequently Alexa555-conjugated tyramide (left, Red channel). The nuclei were visualized as blue.

In summary, since genetically fused target-specific peroxidase can bind various targeting antibodies through binding domain Z, this bifunctional peroxidase is promising as a target-specific signal amplifier.

2.4 Conclusions

In conclusion, the novel fusion proteins, which simultaneously have peroxidase activity and targeting moieties, have been developed as alternative biomolecules of the HRP-conjugated secondary antibody to be used in target-specific signal amplification assay. Other than a glycoprotein, HRP, the APEX2 has advantages of smaller size and less structural complexity that can be easily and largely produced using overexpression system of *E. coli*. To combine with targeting moiety, the ABD from protein A was used to specifically and selectively bind to the various antibodies, originated from mouse, rabbit, rat, and even human. Therefore, the fusion proteins, APEX2-ABD, have been developed. After optimization of the culture conditions of the APEX2-ABD, its soluble forms can be accomplished with a high ratio of the heme incorporation. Also, we can verify the binding ability of the APEX2-ABD, compared with the APEX2 only, using the QCM, SPR, and *in vitro* molecular imaging studies. Furthermore, its catalytic activity was characterized and its efficacy to be used in signal amplification assay was successfully demonstrated using TSA assay. Therefore, we developed the novel fusion protein, APEX2-ABD, which show versatility to bind the numerous antibodies regardless of the origins of the antibodies without thinking pairing between primary and secondary antibody. This suggests a huge potential of the APEX2-ABD as a novel form of target-specific enzyme that could be used in signal amplification assay.





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